CHAPTER 2

Fibroblast growth factor-1 and 2 preserve long-term repopulating ability of hematopoietic stem cells in serum-free cultures

Joyce S. G. Yeoh[1], Ronald van Os[1], Ellen Weersing[1], Albertina Ausema[1], Bert Dontje[1], Edo Vellenga[2], Gerald de Haan[1]

[1] Department of Cell Biology, Section Stem Cell Biology, University Medical Centre Groningen, The Netherlands

[2] Department of Hematology, University Medical Centre Groningen, The Netherlands

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Abstract

In this study we demonstrate that extended culture of unfractionated mouse bone marrow (BM) cells, in serum-free medium, supplemented only with Fibroblast Growth Factor (FGF)-1, FGF-2 or FGF-1+2 preserves long-term repopulating hematopoietic stem cells (HSCs). Using competitive repopulation assays, high levels of stem cell activity were detectable at 1, 3 and 5 weeks after initiation of culture. FGFs as single growth factors failed to support cultures of highly purified $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells. However, co-cultures of purified CD45.1 LSK cells with whole BM CD45.2 cells provided high levels of CD45.1 chimerism after transplant, showing that HSC activity originated from LSK cells. Subsequently, we tested reconstituting potential of cells cultured in FGF-1+2 with the addition of early acting stimulatory molecules, stem cell factor + interleukin-11 + Flt3 ligand. The addition of these growth factors resulted in a strong mitogenic response, inducing rapid differentiation and thereby completely overriding FGF-dependent stem cell conservation. Importantly, although HSC activity is typically rapidly lost after short term culture in vitro, our current protocol allows us to sustain stem cell repopulation potential for periods up to 5 weeks.

Introduction

Hematopoietic stem cells (HSCs) play a vital role in establishing and maintaining hematopoiesis throughout life. Key to all stem cell transplantation therapies is the unique property of HSCs to undergo self-renewal and to functionally repopulate the tissue of origin when transplanted into a myeloablated recipient.

It has been shown that HSCs can undergo a large number of self-renewing divisions in vivo, where the actual number of HSCs can increase. For example, in mice, it has been shown that a single stem cell can regenerate and maintain the entire lymphohematopoietic system after transplantation into an irradiated or immunocompromised host[1-3].

Recent evidence suggests that signaling molecules involved in embryonic development, when the hematopoietic system is first formed, play an important role in regulating stem cell self renewal. These include Wnt, Bmp and Shh family members[4-9]. In a recent study we showed that fibroblast growth factor (FGF)-1 is also a potent stimulator of stem cell activity in vitro[10]. In addition, we showed that all long term repopulating hematopoietic stem cell activity is contained in the lineage-depleted, FGF receptor (FGFR)-positive cell population in mouse bone marrow (BM).

FGF-1 belongs to the family of FGFs of which to date, 22 FGFs and 4 FGF receptors have been identified in vertebrate genomes[11]. All FGFs have a high affinity for heparin and for cell surface heparan sulfate proteoglycan (HSPG)[12]. This complex formation is crucial for high affinity binding of FGF to its receptors. Multiple pleiotropic and overlapping activities of FGF family members have been reported. A large body of evidence from human disorders and gene knockout studies shows that FGF pathways are required for vertebrate and invertebrate development[13-18]. FGFs are also prominent in the development of the limbs[19-21], nervous system[22;23], and angiogenesis[24;25]. Additionally, several members of the FGF family are potent inducers of mesodermal differentiation in early embryos[26]. Interestingly, FGF-2 has been identified as a strong stimulator of human embryonic stem cell (hESC) self-renewal. The addition of FGF-2 to serum-free medium allows the clonal culture of hESCs[27]. Recently, it has been reported that hESC culture can be simplified by using high doses of FGF-2 which are adequate to maintain hESCs long-term under feeder-free and serum-free growth conditions[28-30]. Interestingly, neural stem cells grown in

three dimensional sphere-like structures in vitro also require the presence of FGF-2[31;32].

The role of FGFs for in vitro maintenance of hematopoietic stem cells has remained largely unexplored. In the present study, we compared the growth of HSCs in serum-free medium supplemented with FGF-1 and/or FGF-2. We show that long-term repopulating stem cells can be conserved in vitro for periods up to 5 weeks.

Materials and Methods

Mice

Female C57BL/6 SJL CD45.1 mice, originally obtained from the Jackson Laboratory (Bar Habor, ME, http://www.jax.org) and bred in our local animal facility were used as a donor source of HSCs. C57BL/6-Tg(ACTB-EGFP)10sb/J transgenic green fluorescent protein (GFP) mice originally purchased from The Jackson Laboratory were bred in our local animal facility and also used in certain experiments. Wild type female C57BL/6 mice were purchased from Harlan (Horst, The Netherlands, www.harlaneurope.com) and maintained under clean conventional conditions in the animal facilities of the Central Animal Facilities, University of Groningen (The Netherlands). Mice were fed ad libitum with food pellets and acidified tap water (pH = 2.8). All animal procedures were approved by the local animal ethics committee of the University Medical Centre Groningen.

Hematopoietic Cells

Mice were sacrificed by cervical dislocation and BM cells were obtained by crushing both femora. Marrow cells were resuspended in α-minimum essential medium (α-MEM; Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com) supplemented with 2% fetal calf serum (FCS; Gibco-BRL). The cell suspensions were filtered through a 100μM cell strainer (BD Falcon, Two Oak Park, MA http://www.bdbioscience.com) to remove debris. Cells were counted on a Coulter Counter Model Z2 (Coulter Electronics, Hialeah, FL, http://www.beckmancoulter).

Stem Cell Expansion Culture System

Unfractionated C57BL/6.SJL CD45.1 BM cells were cultured at 5 x 10^6 cells per well in a 6 well plate (Corning Incorporated, Corning, NY, http://www.corning.com) in StemSpan serum free medium (Stem Cell Technologies; Vancouver, Canada, http://www.stemcell.com) in the presence of 10ng/ml recombinant human FGF-1 (Gibco, Grand Island, NY, http://www.invitrogen.com), or with 10ng/ml FGF-2 (Sigma-Aldrich, St Louis, http://www.sigmaaldrich.com), or a combination of both cytokines at 10ng/ml each. Culture media was also supplemented with 10μg/ml heparin (H3149 Sigma-Aldrich). In some experiments, unfractionated C57BL/6.SJL 5.1 cells isolated and cultured with StemSpan serum-free medium, 10μg/ml heparin

and FGF-1+2 were treated with a cocktail of hematopoietic growth factors (GFs). A cocktail of SCF (300ng/ml) (Amgen, Thousand Oaks, CA), interleukin (IL)-11 (20ng/ml) (R&D Systems, Minneapolis, http://www.rndsystems.com) and Flt3 ligand (Flt3L) (1ng/ml) (Immunex, Seattle, http://immunex.com) was added to the cultures for 1, 3 and 5 weeks. Non-adherent cells were harvested weekly, counted to determine growth kinetics and re-introduced into the expansion culture and fresh GFs were added to the culture. At 1, 3 and 5 weeks of culture, non-adherent and adherent cells were harvested and counted in preparation for cell analysis and in vivo transplantation assay into lethally irradiated C57BL/6 mice.

Isolation of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ Cells

Freshly isolated C57BL/6 BM cells were stained with biotinylated lineage-specific antibodies (Mouse Lineage Panel, containing anti-CD45R, anti-CD11b, anti-TER119, anti-Gr-1 and anti-CD3e (BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), FITC-anti-Sca-1 and APC-anti-c-kit (BD Pharmingen). $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells were stained as described[33]. Cells were either analyzed on the FACS Calibur (Becton, Dickinson and Company, San Jose, CA, http:www.bd.com) or sorted by a MoFlow cell sorter (DakoCytomation, Fort Collins, CO, http://www.dako.com).

Cell Analysis

FGF-expanded cells were spun for cytospin preparation. Cytospin preparations were stained with May-Grünwald-Giemsa. Cytospots were washed with distilled water and allowed to air dry before analysis under a microscope.

Levels of chimerism were determined by detecting the presence of GFP or CD45.1 and CD45.2 positive cells in transplanted mice. To detect CD45.1 and CD45.2 positive cells, cells were stained with anti-CD45.2 (FITC) and CD45.1 (phycoerythrin) antibodies (BD Pharmingen) for 30 minutes and analyzed on a flow cytometer (FACS Calibur; Becton, Dickinson and Company).

Cobblestone Area Forming Cell Assays

Cobblestone area forming cell (CAFC) assays were performed as described[34] to assess the number of hematopoietic progenitor cells (day 7 CAFCs) or more primitive stem cells (day 35 CAFCs) in the FGF expanded cultures. Adherent and non-adherent cells

were collected and seeded in limiting dilution in 96-well plates (Corning Incoporated) containing a pre-established fetal bone marrow-derived-1 stromal layer. The cells were cultured in Iscove's modified Dulbecco's modified Eagle's Medium (Gibco-BRL, Paisley, Scotland, http://www.gibcobrl.com) supplemented with 20% horse serum at 34^{o}C in a 10% CO_2 incubator. On a weekly basis, wells were scored and half the volume of the medium was changed. Wells were scored positive if cobblestone areas were present. *P values* were used to test the statistical significance of different groups. The student's *t-test* was used assuming unequal variances of the two variables. The Poisson-based limiting dilution analysis calculation was used with a 95% confidence interval to determine significant differences at $p < .05$. As previously reported, quantification of CAFCs at days 7 and 35 was performed by using maximum likelihood ratio method[35].

In Vivo Transplantation Assays

Female C57BL/6 mice were used to provide competitor cells and as recipient mice. BM cells were obtained by flushing the femoral content 3 times with α-MEM supplemented with 2% FCS. Recipient mice were irradiated with 9.5Gy γ-rays (0.7026Gy/minute) in a IBL 637 Cesium 137 source (CIS bio-international, Gif-sur-Yvette, France, http://www.cisbiointernational.fr), 24 hours prior to transplantation. For competitive repopulation determination, varying doses of cultured BM cells were mixed with a constant number of BM competitor cells. Thus, recipient mice were intravenously transplanted with different dilutions of expanded stem cells, with or without 2 x 10^5 life-sparing C57BL/6 BM competitor cells. Each transplant group consisted of 6 recipients. After transplantation, blood samples (60μl) were taken monthly from the retro-orbital sinus for flow cytometer analysis. At the time of sacrifice, chimerism in BM samples was analyzed by fluorescence-activated cell sorting (FACS) in the same manner. For each recipient, the competitive repopulating index (CRI) was determined. CRI is a relative measure of the competitive ability of cultured cells in comparison with that of fresh BM cells. The CRI was calculated by using the following formula:

$$\text{CRI} = \frac{\text{Ratio of cultured cells to competitor cells in the circulation}}{\text{Ratio of cultured cells to competitor cells transplanted}}$$

A CRI value of 1 indicates by definition that cultured cells and competitor cells have equal competitive ability. The repopulation ability of our cells can also be measured in repopulation units (RU). The RU takes into account the total number of cells generated. Each RU is equivalent to the repopulation function of 100 000 competitor BM cells. The RU was calculated using the following formula:

$$\text{RU} = (\text{Ratio of cultured cells to competitor cells in the circulation}) \text{ x No. of competitor RU}$$

$$\text{RU/well} = (\text{Number of RU}) \text{ x } \frac{(\text{Total number of cultured cells per well})}{(\text{Number of transplanted cells})}$$

Results

Cell Growth Kinetics and Cell Morphology

We used FGF-1 and FGF-2 separately and the combination of both FGFs as the only stimulus in serum-free media to culture CD45.1 BM cells for a period up to 5 weeks. One and 3 weeks following initiation of culture, a decrease in the number of cells was observed. The number of cells per well had dropped dramatically from 5 x 10^6 to an average of approximately 5 x 10^5 cells for all FGF conditions (Figure 2.1A). Five weeks after the initiation of culture, cells treated with FGF-1 and/or FGF-2 had increased close to the input cell number, whereas cells cultured only in serum-free media remained low throughout the 5-week culture period at 1 x 10^5 (Figure 2.1A). As a first screen test, prior to in vivo assays, in vitro CAFC assays were setup. CAFC subsets were quantified in cells harvested from the FGF-1+2 expansion cultures at 3 and 5 weeks. The absolute number of day 7 CAFCs that were harvested from 3-week cultures had increased 1.5- fold (Figure 2.1B). Interestingly, a substantial 26-fold expansion of day 7 CAFCs was observed at the 5 week culture time point. In contrast, day 35 CAFCs at week 3 cultures, were slightly lower than input, whereas a 3.5-fold expansion was apparent after 5 weeks of culture (Figure 2.1B).

May-Grunwald-Giemsa staining of the starting cell population (Figure 2.1C) and of FGF-1+2-treated cells showed an accumulation of macrophages and blast-like cells after 3 and 5 weeks of culture (Figure 2.1D and 2.1E). The presence of blast-like cells and extensive CAFC activity indicated the possible existence of immature cells with stem cell properties in FGF stimulated cultures.

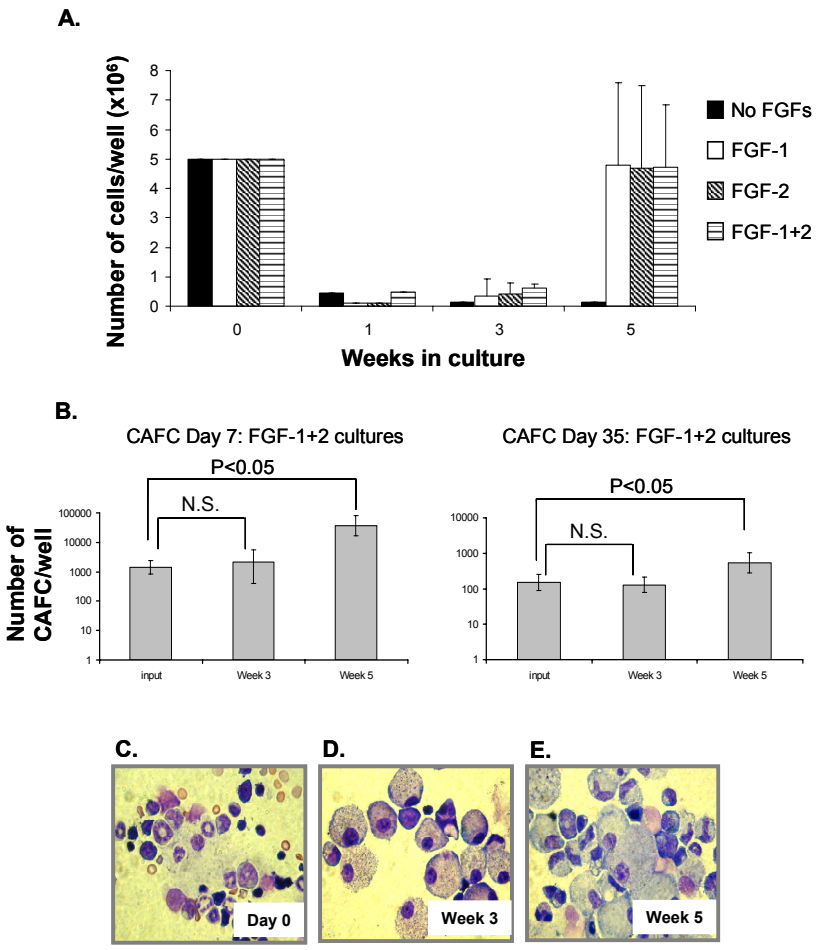

Figure 2.1: Cell growth, morphology and phenotype of cells. (A): Growth kinetics of unfractionated bone marrow (BM) cells in culture for 5 weeks. These are 4 representative cultures out of 30 cultures. Some were performed in 25cm^2 flasks; the remaining in 6-well plates. Cells were cultured in serum-free medium, supplemented with FGF-1, or FGF-2, or FGF-1+2 in the presence of 10µg/ml heparin. (B): Unfractionated BM cells and cells cultured for 3 and 5 weeks with FGF-1+2, were placed in limiting dilutions in a 96-well plate and absolute numbers of day 7 and 35 CAFCs were compared with input cells. The input values refer to freshly isolated untreated bone marrow cells. Day 7 and 35 CAFC activity was higher for cells after a culturing period of 5 weeks with FGF-1+2; p-values were calculated using Student's t test and Poisson-based limiting dilution analysis was used to determine the CAFC frequency. (C): May-Grünwald-Giemsa staining was performed on BM cells cultured in the presence of FGF-1+2 at day 0 of initiation of culture. (D): May-Grünwald-Giemsa staining was performed on BM cells cultured in the presence of FGF-1+2 at 3 weeks after initiation of culture. (E): May-Grünwald-Giemsa staining was performed on BM cells cultured in the presence of FGF-1+2 at 5 weeks after initiation of culture; N.S., not significant.

Long-Term Competitive In Vivo Reconstitution of FGF Expanded Cells

To assess whether cells cultured with FGF-1, FGF-2 or FGF-1+2 contained stem cell activity, we competitively transplanted congenic B6.CD45.1 or transgenic B6.GFP$^+$ FGF expanded cells with CD45.2 BM cells at 1, 3 and 5 weeks after the initiation of culture. In each group of transplants, 6 recipient mice were transplanted. Animals receiving 1 week cultured cells were transplanted with 2.5 x 10^5 cultured cells and 2.5 x 10^5 B6 CD45.2 BM cells. As shown in Figure 2.2A and Table 2.1, after 1 week of culturing, the CRI of FGF-1+2 cultured cells, was approximately 50, 18 weeks after transplant. Interestingly, CRI levels of FGF-1+2 cultured cells increased with time, suggesting engraftment of relatively more cells with long-term repopulation ability. Average chimerism levels after 18 weeks were 96% (Table 2.1). After 3 weeks of culturing, recipient mice received 1.8 x 10^5, 2.2 x 10^5, and 3.5 x 10^5 FGF-1, FGF-2 and FGF-1+2 cultured cells, respectively, together with 2 x 10^5 B6 CD45.2 competitor cells. Chimerism levels of ~80% were achieved, corresponding to a CRI level of approximately 5, 16 weeks post-transplant. No significant differences were observed between the FGF groups (Figure 2.2B and Table 2.1). As expected, cell cultured for 3 and 5 weeks in serum-free medium without any supplements had no long-term reconstituting activity. Remarkably, 5 weeks after culturing 1.2 x 10^6 FGF-2 and 1.1 x 10^6 FGF-1+2 cells still outcompeted 2 x 10^5 freshly isolated BM cells, although CRI values dropped significantly compared to cells cultured for 1 or 3 weeks (Figure 2.2C; Table 2.1). Low CRI values of 0.5 ± 0.2 for FGF-1 cultured cells were obtained, suggesting that these cells provide little competitive repopulation ability (Figure 2.2C) even though 1.62 x 10^6 cells were transplanted with 2 x 10^5 B6 CD45.2 BM cells. The reconstitution activity of FGF cultured cells as indicated by the total number of repopulation units/well (RU) correlates with the CRI values (Figure 2.2D; Table 2.1). A 5-fold increase in RU was observed with cells treated with FGF-1+2 for 1 week. After 5 weeks of culturing, both FGF-2 and FGF-1+2 cultured cells had a 1.5-fold increase in RU compared to input cells (Figure 2.2D; Table 2.1).

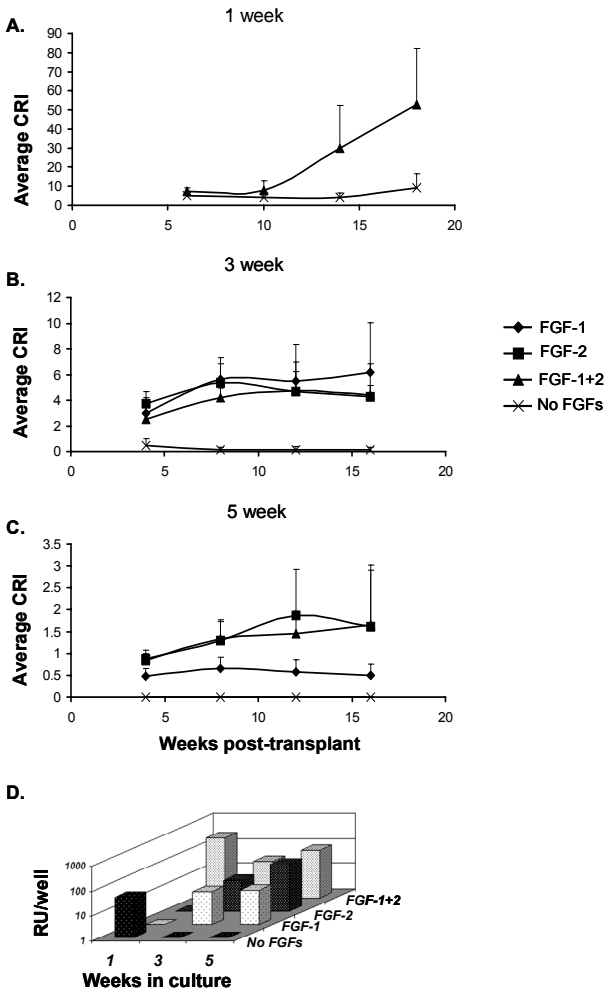

Figure 2.2: In vivo competitive transplantation assay of FGF cultured cells. (A): B6 CD45.1 cells cultured in serum-free medium alone, or in the presence of FGF-1, FGF-2 or both FGF-1+2 were transplanted into lethally irradiated B6 CD45.2 recipient mice after 1 week of culture. (B): B6 CD45.1 cells cultured in serum-free medium alone, or in the presence of FGF-1, FGF-2 or both FGF-1+2 were transplanted into lethally irradiated B6 CD45.2 recipient mice after 3 weeks of culture. (C): B6 CD45.1 cells cultured in serum-free medium alone, or in the presence of FGF-1, FGF-2 or both FGF-1+2 were transplanted into lethally irradiated B6 CD45.2 recipient mice after 5 weeks of culture. For week 1 culture, mice were transplanted with 2.5 x 10^5 cultured cells and 2.5 x 10^5 B6 CD45.2 bone marrow (BM) cells. Recipients receiving 3 weeks cultured cells were transplanted with 1.8 x 10^5 FGF-1, 2.2 x 10^5 FGF-2, 3.5 x 10^5 FGF-1+2 and 7.5 x 10^4 no FGF treated cells with 2 x 10^5 B6 CD45.2 BM cells. 5 weeks after culturing 1.6 x 10^6 FGF-1, 1.2 x 10^6 FGF-2, 1.1 x 10^6 FGF-1+2 and 4.8 x 10^5 no FGF treated cells were transplanted into recipient mice with 2 x 10^5 B6 CD45.2 BM cells. Average CRI ±

SD was calculated in each group consisting of 6 mice. (D): The absolute number of repopulating units of cultured cells was compared to input cells. Each RU is equivalent to the repopulation function of 100,000 competitor bone marrow cells. Therefore, at initiation of culture, 5 x 10^6 whole BM cells contain 50 RU. Following 1 week of culturing in FGF-1+2, 253 RU were generated. Both FGF-2 and FGF-1+2 cultured cells after 5 weeks produced 75 RUs. Abbreviations: CRI, competitive repopulation index; FGF, fibroblast growth factor; RU, repopulation unit.

Table 2.1 In vivo reconstitution of FGF expanded cells

	No. cells/well	Number of cells at end of culture (x10^6/well)			No. of CD45.1 cultured cells transplanted			% CD45.1 chimerism 16-18 weeks post-transplant			CRI			RU/well			
	Day 0	Week 1	Week 3	Week 5	Week 1	Week 3	Week 5	Week 1	Week 3	Week 5	Week 1	Week 3	Week 5	Day 0 *	Week 1	Week 3	Week 5
No FGFs	5×10^6	0.45 ± 0	0.13 ± 0	0.13 ± 0	2.5×10^5	7.5×10^4	1.6×10^5	89 ± 16	4 ± 6	0.2 ±0	9 ± 7	0.12 ± 0.2	0	50	41.5 ± 33	0.16 ± 0.3	0
FGF-1	5×10^6	0.1 ± 0	0.33 ± 0.6	4.8 ± 2.8	2.5×10^5	1.8×10^5	1.6×10^6	N.D.	82 ± 8	77 ± 10	N.D.	6.2 ± 3.9	0.5 ± 0.3	50	N.D.	20 ± 13	24 ± 12
FGF-2	5×10^6	0.1 ± 0	0.4 ± 0.4	4.7 ± 2.8	2.5×10^5	2.2×10^5	1.2×10^6	N.D.	78 ± 10	85 ± 11	N.D.	4.3 ± 2.6	1.6 ± 1.3	50	N.D.	17 ± 10	75 ± 60
FGF-1+2	5×10^6	0.46 ± 0	0.63 ± 0.1	4.7 ± 2.1	2.5×10^5	3.5×10^5	1.1×10^6	96 ± 4.6	88 ± 1.8	87 ± 5.6	53 ± 29	4.4 ± 0.8	1.7 ± 1.4	50	253 ± 173	28 ± 10	79 ± 64

The data presented in this table were used to generate Figures 2.1 and 2.2. All mice were transplanted in competition with 2 x 10^5 B6 CD45.2 BM cells. N.D. indicates that the experiment was not performed.

* Each RU is equivalent to the repopulation function of 100,000 competitor bone marrow cells. Therefore, at day 0, 5 x 10^6 whole BM cells contain 50 RU

Abbreviations: CRI, competitive repopulation index; FGF, fibroblast growth factor; N.D., experiment not performed; RU, repopulation unit.

Radioprotection and Long-Term In vivo Reconstitution of FGF-Expanded Cells

To test the ability of expanded cells in a more clinically relevant model, 1 x 10^4 and 5 x 10^4 FGF-1+2 expanded cells were transplanted in lethally irradiated C57BL/6 mice without competitor cells. After 3 weeks of culture, 1 x 10^4 and 5 x 10^4 cells were able to stably engraft into most recipient mice providing long-term repopulation. Transplantation of 1 x 10^4 cells provided radioprotection to 60% of animals (Figure 2.3A). This value increased to 80% when 5 x 10^4 FGF-1+2 expanded cells were transplanted (Figure 2.3A). The survival rate was higher when culture time was extended to 5 weeks with 1 x 10^4 expanded cells providing radioprotection to 80% of animals (Figure 2.3A). A further increase in survival was observed with 5 x 10^4 expanded cells providing radioprotection to 100% of the mice (Figure 2.3A). In all cases a radioprotection endpoint of 4 weeks after transplant was used. Animals that died of hematopoietic failure, died within 14 days post-transplant. Transplantation of 1 x 10^4 cells cultured for 3 weeks resulted in an average chimerism of 50%, whereas 5 x 10^4 cells engrafted with an average chimerism of 75%, 28 weeks after transplant (Figure 2.3B). Chimerism results from transplants carried out with cells from 5 week

cultures are shown in Figure 2.3C. With 1 x 10^4 FGF-1+2 expanded cells, engraftment levels steadily increased, stabilized after 8 weeks and 26 weeks after transplant donor contribution was more than 90%. Mice transplanted with 5 x 10^4 cells showed an average level of chimerism of more than 95%.

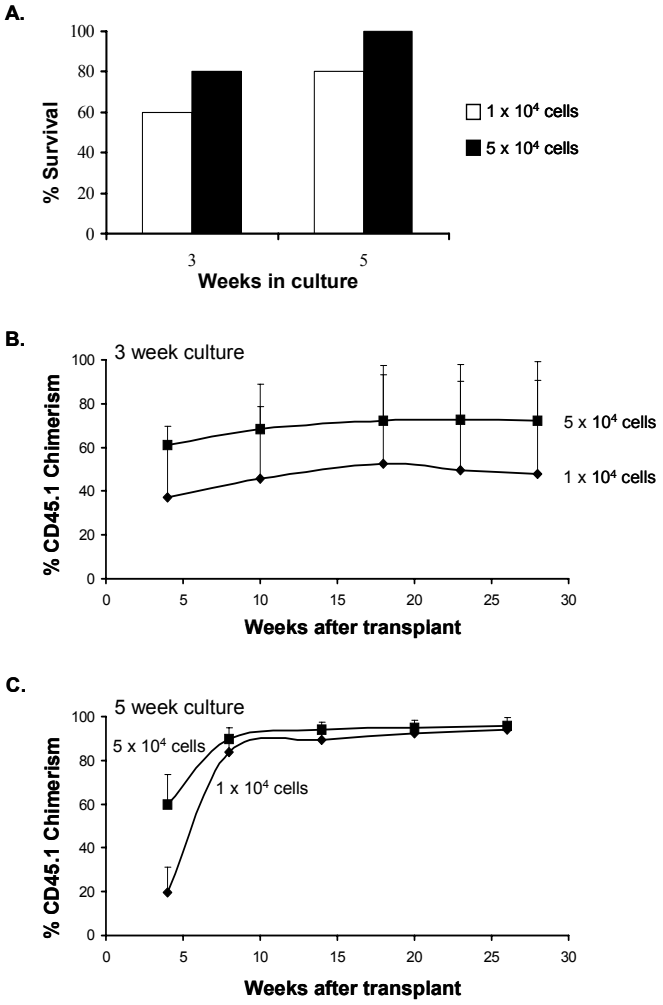

Figure 2.3: Radioprotective potential of fibroblast growth factor (FGF)-expanded cells. (A): Survival rate of mice transplanted only with expanded cells. (B): Unfractionated B6 CD45.1 bone marrow (BM) cells were cultured in FGF-1+2 for 3 weeks. (C): Unfractionated B6 CD45.1 bone marrow (BM) cells were cultured in FGF-1+2 for 5 weeks. Recipient CD45.2 C57BL/6 mice were lethally irradiated and transplanted with 1 x 10^4 or 5 x 10^4 FGF-1+2 cultured cells without life sparing competitor cells. Analysis of chimerism was performed and average results are shown.

Growing of Lin-Sca-1$^+$c-Kit$^+$ Cells in Cocultures

In our 3 and 5 week FGF cultures described above, the percentage of LSK cells was analyzed prior to transplantation. After 3 weeks of culturing LSK frequencies of FGF-1 cultured cells was 0.7% whilst FGF-2 and FGF-1+2 cells were 1.3%. LSK frequencies increased after 5 weeks of culturing to 0.9% for FGF-1 cells and 2.8% for FGF-2 and FGF-1+2 cells (data not shown). Despite the high percentage of LSK cells in these cultures compared to normal BM cells which have a LSK frequency of ~0.2%, we were not successful at culturing purified LSK HSCs or bulk Lin$^+$ and Lin$^-$ cells in serum-free medium supplemented with FGF-1+2 (data not shown). Thus, we speculated that either the stem cell growth in unfractionated BM cultures did not originate from LSK cells, or alternatively, that an accessory population of cells contained within the bone marrow was required. To test this hypothesis, we sorted LSK cells from B6 CD45.1 mice and co-cultured 7 x 10^3 and 5 x 10^4 of these cells in the presence of 5 x 10^6 CD45.2 unfractionated BM cells. FACS analysis showed that 5 x 10^6 unfractionated CD45.2 BM cells contained ~5,500 LSK cells. Purified stem cells and whole BM were cocultured for 5 weeks. After culture, all cells were harvested and 2 x 10^5 cells were transplanted into lethally irradiated recipients without competitors. The percentage of white blood cells originating from the purified LSK CD45.1$^+$ fraction or from the CD45.2$^+$ unfractionated cells was assessed in the recipients. If only LSK cells were responsible for the FGF stimulated stem cell activity in unfractionated BM, we would expect chimerism levels of the sorted 7,000 CD45.1 LSK cells to reach or come close to 60% (7,000 CD45.1 LSK cells + 5500 LSK CD45.2 cells: 7,000/(7,000+5,500) = 56%). Strikingly, 16 weeks post-transplant, chimerism levels had increased to 51%, 52% and 64%, implying that indeed all FGF induced stem cell activity is derived from the LSK population, (Figure 2.4A). Chimerism levels in recipients transplanted with 5 x 10^4 CD45.1 LSK cultured in 5 x 10^6 CD45.2 unfractionated BM ranged from 70% to 99%, 16 weeks after transplant (Figure 2.4B). Engraftment was seen in all mice transplanted. The estimated expected level of chimerism in these animals was 50,000/(50,000+5,500) = 90%, clearly well in range with the experimental findings. These results suggest that FGFs may be acting both on LSK cells and on other cell types indirectly affecting LSK cells to induce stem cell activity in vitro.

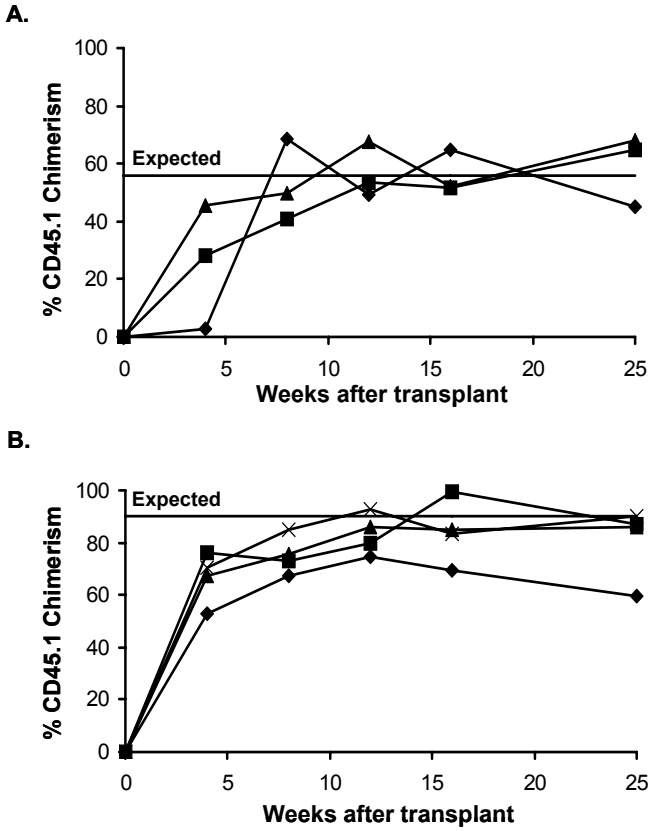

Figure 2.4: Highly purified CD45.1 $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells co-cultured with unfractionated CD45.2 bone marrow (BM) cells. (A): Seven thousand B6 CD45.1 LSK cells were cocultured with 5 x 10^6 B6 CD45.2 whole bone marrow cells (estimated to contain 5500 CD45.2 LSK cells) for 5 weeks in serum-free medium supplemented with fibroblast growth factor (FGF)-1+2. Subsequently, 2 x 10^5 cultured cells were transplanted into B6 CD45.2 mice without additional competitor cells. At start of the culture, the percentage of CD45.1 LSK cells in the co-culture was 7,000/(7,000+5500) = 56%. 12 weeks after transplant, chimerism levels of all mice steadily increased to an average of 57%. (B): Five week coculture of 50,000 B6 CD45.1 LSK and 5 x 10^6 B6 CD45.2 whole BM cells was set-up and similarly transplanted. The percentage of CD45.1 LSK cells in this culture was 50,000/(50,000+5,500) = 90%. Chimerism levels in transplanted recipients rapidly increased reaching 83%. Chimerism levels for each transplanted recipient are denoted by different symbols.

Effect of SCF, IL-11 and Flt3L on FGF-1+2 Induced Clonogenic Activity In Vitro

Although single LSK cells cultured in FGF-1+2 did not divide, they did remain visible for 7 days (data not shown). Thus it appeared that the addition of FGF-1+2 to cell cultures prolonged the lifespan of the cells, but did not induce a strong enough mitogenic signal. More classical hematopoietic GFs such as SCF, IL-11 and Flt3L, have a much stronger proliferating effect and are shown to maintain stem cells in short-term cultures[36]. Therefore we cultured cells with a cocktail of SCF, IL-11 and Flt3L with or without the addition of FGF-1+2. After 5 weeks of culturing, cell numbers of GF treated cells had exponentially increased from 5 x 10^6 to 7 x 10^8, similar to GF + FGF-1+2 cultured cells (Figure 2.5A; Table 2.2). We next tested whether FGFs would be able to maintain stemness of cells when used in combination with SCF, IL-11 and Flt3L, which provides stronger mitogenic signals.

CAFC assays were carried out to determine clonogenic activity of cells treated with a cocktail of growth factors. We observed a significant ($p < .05$) increase in day 7 CAFC activity after 3 weeks of culturing in GFs (Figure 2.5B). CAFC day 7 numbers for cells cultured in the presence of GFs and GF + FGF-1+2, increased 222- and 273-fold, respectively, over the input value, whereas cells treated with FGF-1+2 had a modest 1.5-fold increase over the input value. A contrasting pattern was observed when primitive day 35 CAFC numbers were evaluated (Figure 2.5B). Day 35 CAFCs of 3 week GFs alone, or GF + FGF-1+2 cultures were below the detection level (less than 1 CAFC per 1x10^6). Nevertheless, FGF-1+2 cultured cells were able to maintain day 35 CAFC activity.

Effect of GFs on FGF-1+2 Induced Stem Cell Activity In Vivo

Finally, we determined whether cells cultured in FGF-1+2 with or without SCF, IL-11 and Flt3L provided engraftment in vivo. To this end, varying cell doses ranging from 1.8 x 10^5 to 2 x 10^6 B6 CD45.1 cultured cells were transplanted in competition with 2 x 10^5 freshly isolated B6 CD45.2 BM cells and compared with results for transplanted FGF-1+2 cells (Figure 2.2). Although highly elevated CRI levels were observed 16 weeks after transplant, when FGF-1+2 treated cells were cultured for 1 week, the CRI had dropped to 1 for cells cultured with GFs alone or GF+FGF-1+2 treated cells (Figure 2.5C; Table 2.2). Continued culturing of cells for 3 and 5 weeks decreased CRI values for all conditions. All competitive repopulation ability was lost after 3

weeks of culturing or cells treated with GF alone, or with the addition of GF + FGF-1+2 (Figure 2.5C; Table 2.2).

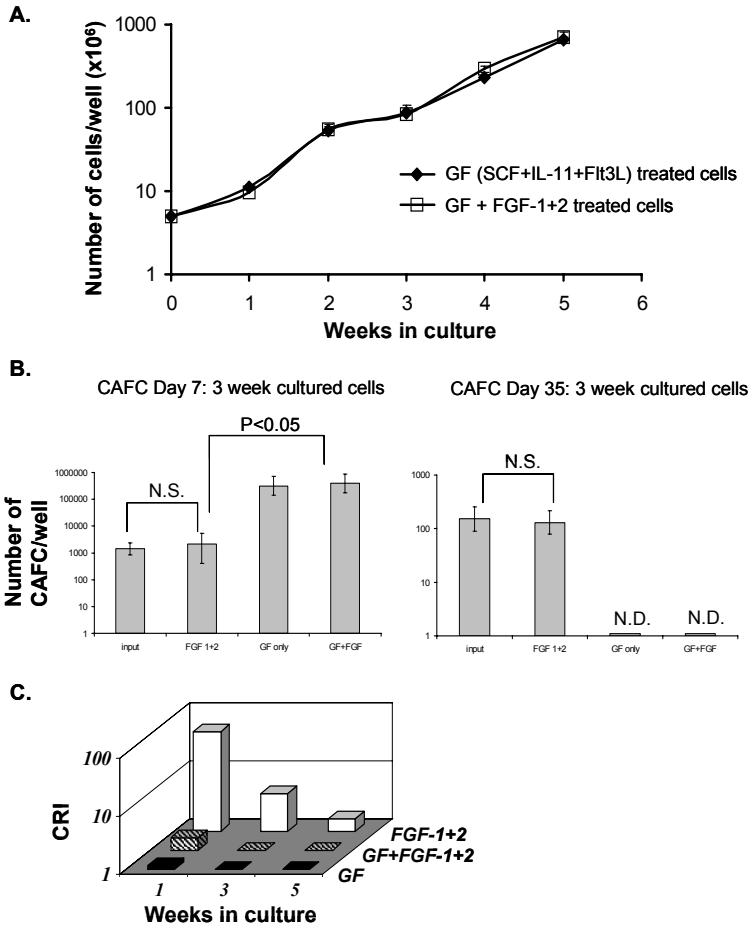

Figure 2.5: Effects of GF treatment on FGF-cultured cells. (A): Growth kinetics of unfractionated bone marrow (BM) cells cultured with a cocktail of GFs (SCF, IL-11 and Flt3L) in the presence or absence of FGF-1+2. Cell growth exponentially increased from 5 x 10^6 to 7 x 10^8. (B): Day 7 and 35 CAFC content of unfractionated BM cells and cells cultured for 3 weeks with FGF-1+2, GFs (SCF, IL-11 and Flt3L) alone and GFs + FGF-1+2. No day 35 CAFC activity was observed for cells cultured with GFs alone and GFs + FGF-1+2; *p*-values were calculated using Student's *t* test, and Poisson-based limiting dilution analysis was used to determine the CAFC frequency. (C): CRI ± SD values (shown in Table 2) for mice transplanted with varying cell doses of FGF-1+2, GF and GF + FGF-1+2 cultured cells in competition with 2 x 10^5 B6.CD45.2 cells. Data are the same as in Figure 2 for FGF-1+2 and are only included for comparison. Abbreviations: CAFC, cobblestone area-forming cell; CRI, competitive

repopulation index; FGF, fibroblast growth factor; Flt3L, Flt3 ligand; GF, growth factor; IL, interleukin; N.D., not detected; N.S., not significant; SCF, stem cell factor.

Table 2.2: Effect of the addition of stem cell factor, interleukin-11 and Flt3 ligand on FGF-1+2 induced stem cell activity

	No. cells in culture	Number of cells at end of culture (x10^6/well)			No. of CD45.1 cultured cells transplanted			% CD45.1 chimerism 16-18 weeks post-transplant			CRI			RU/well			
	Day 0	Week 1	Week 3	Week 5	Week 1	Week 3	Week 5	Week 1	Week 3	Week 5	Week 1	Week 3	Week 5	Day 0 *	Week 1	Week 3	Week 5
FGF-1+2	5×10^6	0.46 ± 0	0.63 ± 0.1	4.7 ± 2.1	2.5×10^5	3.5×10^5	1.1×10^6	96 ± 4.6	88 ± 1.8	87 ± 5.6	53 ± 29	4.4 ± 0.8	1.7 ± 1.4	50	253 ± 173	28 ± 10	79 ± 64
GFs	5×10^6	11 ± 0.8	87 ± 20	652 ± 42	2.0×10^6	2.0×10^6	2.0×10^6	43 ± 24	1.6 ± 1	0	1.2 ± 1.4	0	0	50	45 ± 53	1.4 ± 0.9	0
GFs + FGF-1+2	5×10^6	9.8 ± 1.1	84 ± 13	695 ± 123	2.0×10^6	2.0×10^6	2.0×10^6	52 ± 27	1.4 ± 0.95	0	1.6 ± 1.1	0	0	50	71 ± 49	1.23 ± 0.8	0

The data on this table were used to generate Figure 2.5. Results shown for FGF-1+2 condition are also presented in Table 1 and are added for comparison. All recipients were transplanted in competition with 2 x 10^5 B6 CD45.2 bone marrow (BM) cells.

* Each RU is equivalent to the repopulation function of 100,000 competitor bone marrow cells. Therefore, at day 0, 5 x 10^6 whole BM cells contain 50 RU

Abbreviations: CRI, competitive repopulation index; FGF, fibroblast growth factor; GF, growth factor; RU, repopulation unit.

Discussion

In the present study, we tested the potential of different FGFs to support HSC growth in serum-free medium. In all 3 FGF conditions, FGF-1, FGF-2 and FGF-1+2, similar trends in overall cell growth and cell morphology were observed. Although not evident from the growth and morphology of the cultured cells, the addition of FGF-1 and/or -2 to serum-free medium proved to be an effective culture condition to support primitive HSCs. Our in vitro CAFC data and in vivo reconstitution results clearly document that bona fide long-term repopulating stem cells can be preserved in vitro for up to 5 weeks when FGFs are added to the medium (Figure 2.1 and 2.2).

To test the repopulating potential of FGF-treated stem cells in a clinically relevant model, cultured cells were transplanted into lethally irradiated mice without competitor cells. Importantly, BM cells cultured for 3 and 5 weeks in the presence of only FGF-1+2 were able to provide radioprotection and reconstitution.

Thus, our data clearly document that FGFs (of which we tested 2 out of a family of 22) can be added to a growing list of signaling proteins that act on primitive stem cells. Interestingly, whereas "classical" hematopoietic growth factors turn out to have limited potential in sustaining and expanding HSCs[37-40] the effect of growth factors and morphogens that historically have been associated with embryonic development (Wnt, BMP, FGF, Shh, and insulin-like GF [IGF]-2) may be more powerful[4;7;41;42]. Recently it was reported that FGF-2 allows the clonal growth of hESCs in medium containing serum replacement[27]. In addition, ESCs are known to express multiple FGF receptors[43]. Xu et al, documented that FGF-2 synergizes with Noggin to suppress BMP signaling and thus sustain undifferentiated proliferation of hESCs in the absence of fibroblasts or conditioned medium[29]. It is also interesting to note that during development TGF-β/BMP, IGF, Notch receptors and Wnts may be influenced and dependent on heterologous factors such as FGFs[44]. Lately , it was reported that cross-talk between the Notch and FGFR signaling pathways may be an important auto-regulatory mechanism involved in the regulation of cell growth[45]. Also, FGFs and Wnts have been shown to interact in a variety of developmental systems including tracheal development in *Drosophila*, mesoderm induction in Xenopus, and brain, tooth, and kidney development in other vertebrates[46]. FGF signaling maintains the proliferation of multipotent neural stem cells and also affects subsequent lineage commitment during neural differentiation. It was shown that these FGF effects are

mediated by β-catenin[47]. Collectively, these data imply an important physiological role for FGF in stem cell fate decisions. The specific biological response, such as proliferation, apoptosis, and differentiation, that a cell will deliver in response to FGF signals, will depend on the interaction with many other factors[44].

We were unable to culture purified LSK cells with only FGFs, whilst recently Zhang and Lodish were able to culture purified BM side population cells with a greater than 8-fold increase in repopulating HSCs when grown in low levels of SCF, thrombopoietin (TPO), IGF-2 and FGF-1 in serum-free medium for 10 days[48]. In our purified cell culture system, such cross-talk with other signaling networks was not possible, suggesting that the combination of SCF, TPO, IGF-2 and FGF-1 are better suited for the expansion of a purified population of HSCs. The results of Zhang and Lodish and our results highlight the importance of FGF in an in vitro culture system to maintain HSCs, however our findings also suggests that the effect of FGFs on stem cells requires other stimuli. The addition of SCF, IL-11 and Flt3L increased the proliferation of stem cells. We tested whether FGFs were able to maintain the primitiveness of stem cells, thereby negating the potential differentiation effect of these GFs when placed in combination. Thus, GF + FGF-1+2 cultured cells would have been expected to provide competitive reconstitution whereas GF-only cultures would have been expected to provide no or very little reconstitution. Unfortunately, this was evidently not the case (Figure 2.5). The optimal combination of GFs required for maintaining primitive HSC activity remains to be discovered.

Our studies were not aimed to delineate the molecular consequences of incubating BM cells with FGFs. Consequently, we can only speculate on how FGFs maintain stem cells in whole BM cultures. It has been shown that receptors for FGF-1 are present on primitive hematopoietic cell subsets[10]. Therefore, we speculate that FGFs maintain stem cells at least partially by acting directly on FGFRs expressed on the stem cells. Additionally, other non-LSK cell types normally present in the stem cell niche may carry FGFRs and therefore be responsive to FGFs and may play an important role in the maintenance of stem cells. In the intact animal, stem cells are found in close association with their cellular microenvironments[49-51]. These observations suggest both the existence of stem cell niches[6;52-54] and the notion that in vivo stem cell regulatory mechanisms are likely to require cell-cell contact or short-range interactions[55], providing further evidence that additional stimuli may be required for a desired effect. The niche is composed of stem cells and a diverse

variety of neighboring hematopoietic and non-hematopoietic cell types such as osteoblasts and endothelial cells. These act to secrete and organize a rich milieu of extracellular matrix and other factors that allow stem cells to manifest their unique intrinsic properties[56]. We speculate that for HSC to be properly maintained and amplified in vitro, the whole BM in co-culture must act as a niche, facilitating stem cells to expand.

It is tempting to postulate that many stem cell expansion studies have not been so successful, because cultures almost invariably were initiated with purified cells. As shown in our purification studies, disruption of HSC from their niche is likely to have detrimental effects on their subsequent developmental potential. Our study represents a clear example of an in vitro system using FGFs, capable of supporting primitive HSCs for an extended period of time. Future studies will be aimed at creating a niche for stem cells in vitro, while stimulating their proliferation.

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